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(54) Title: MICROBIAL PREPARATION OF SUBSTANCES FROM AROMATIC METABOLISM/I

(57) Abstract

The invention makes available, by means of an increased provision of intracellular metabolic intermediates, in particular of erythrose 4–phosphate, alternative processes for the microbial preparation of substances, in particular of aromatic amino acids such as L–phenylalanine, in which processes the activity of a transaldolase is increased in a microorganism producing these substances. In preferred embodiments of the invention, the activity of a transketolase or the activity of a transport protein for the PEP–independent uptake of a sugar and/or the activity of a kinase which phosporylates the relevant sugar are/is additionally increased. The invention also relates to gene structures, and to transformed cells carrying these gene structures, which make it possible to implement these processes in a particularly successful manner.

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MICROBIAL PREPARATION OF SUBSTANCES
FROM AROMATIC METABOLISM/I

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The invention relates to a process for the
microbial preparation of substances, in particular
aromatic amino acids, according to Claims 1-23, 36 and 37,
to gene structures according to Claims 24-29, and to

10   transformed cells according to Claims 30-35.

Microbially prepared substances, such as fine
chemicals, in particular aromatic amino acids, are of
great economic interest, with the requirement for amino
acids, for example, continuing to increase. Thus,

15   L-phenylalanine, for example, is used for preparing
medicaments and, in particular, also in the preparation of
the sweetener aspartame (α-L-aspartyl-L-phenylalanine
methyl ester). L-tryptophan is required as a medicament
and as an additive to animal feeds; there is likewise a

20   need for L-tyrosine as a medicament and also as a raw
material in the pharmaceutical industry. In addition to
isolation from natural materials, biotechnological
preparation is a very important method for obtaining amino
acids in the desired optically active form under

25   economically justifiable conditions. Biotechnological
preparation is effected either using enzymes or using
microorganisms. The latter, microbial, preparation enjoys
the advantage that simple and inexpensive raw materials
can be employed. Since the biosynthesis of amino acids in

30   the cell is controlled in a wide variety of ways, however,
a large number of attempts have already been made to
increase product formation. Thus, amino acid analogues,
for example, have been employed in order to switch off the
regulation of biosynthesis. For example, mutants of

35   Escherichia coli permitting an increased production of L-
phenylalanine were obtained by selecting for resistance to
phenylalanine analogues (GB-2,053,906). A similar strategy

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also led to overproducing strains of *Corynebacterium*
(JP-19037/1976 and JP-39517/1978) and *Bacillus*
(EP-0,138,526). Furthermore, microorganisms which have
been constructed using recombinant DNA techniques are
5   known in which the regulation of biosynthesis is likewise
abolished, with the genes which encode key enzymes which
are no longer subject to feedback inhibition being cloned
and expressed. As a prototype, EP-0,077,196 describes a
process for producing aromatic amino acids in which a 3-
10 deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP
synthase) which is no longer subject to feedback inhi-
bition is overexpressed in *E. coli*. EP-0,145,156 describes
an *E. coli* strain in which chorismate mutase/prephenate
dehydratase are additionally overexpressed for the purpose
15 of producing L-phenylalanine.

A feature common to the abovementioned
strategies is that the intervention for improving
production is restricted to the biosynthesis pathway which
is specific for the aromatic amino acids. However, in
20 order to increase production still further, efforts must
be made to improve the provision of the primary
metabolites, phosphoenolpyruvate (PEP) and erythrose 4-
phosphate (Ery4P), which are required for producing
aromatic amino acids.

25   PEP is an activated precursor of the glycolysis
product pyruvate; Ery4P is an intermediate in the pentose
phosphate pathway.

Various attempts have been made to achieve a
specific improvement in the provision of the said primary
30 metabolite Ery4P. An increased flow of carbon through the
pentose phosphate pathway was achieved in *E. coli* by
switching off the enzyme phosphoglucose isomerase; this
resulted in tryptophan being formed (Mascarenhas D. et
al., Appl. Environ. Microbiol. 57 (1991) 2995-99).

US-A-5,168,056 disclosed that overexpression of a trans-
ketolase, achieved by means of recombinant DNA techniques,
makes it possible to obtain an increased provision of
Ery4P and, as a consequence, an improvement in the

5   formation of L-tryptophan, L-tyrosine or L-phenylalanine
as products. Both Draths K.M. et al. (J. Am. Chem. Soc.
114 (1992) 3956-62) and Flores N. et al. (Nat. Biotechnol.
14 (1996) 620-3) demonstrated the same effect. However, as
Feldmann has shown, simply increasing the activity of

10   transketolase in *Zymomonas mobilis* strains lacking
transaldolase activity leads to the enrichment of
metabolites of the pentose phosphate pathway in the cells
and, as a consequence, to negative effects on cell growth
(Feldmann S.D. et al., Appl. Microbiol. Biotechnol. 38

15   (1992) 354-61). This physiological effect may possibly be
attributable to an excessive intracellular concentration
of sedoheptulose-7-phosphate. The inventors have also
found a corresponding inhibition of biomass growth as a
consequence of transketolase overexpression in the case of

20   *E. coli* tal· strains.
              The object of the invention is, therefore, to
make available an alternative process for producing
substances, in particular aromatic amino acids, which
process is distinguished by an increased provision of

25   intracellular metabolic intermediates for the synthesis of
these substances without suffering from the above-cited
disadvantages of the processes, which disadvantages are
the consequence of only increasing the activity of the
transketolase.

30   Surprisingly, this object is achieved, according
to the invention, by making available a process for the
microbial preparation of substances, in which process the
activity of a transaldolase is increased in a micro-
organism which is producing these substances, with the

activity of a phosphoenolpyruvate (PEP)-dependent sugar-uptake system in this microorganism being present, reduced or absent.

5   This result is particularly surprising in that it is in no way self-evident that transaldolases play an important role in the growth of microorganisms and in their production of substances. This particularly applies in the case of growth on hexoses. Thus, yeast strains are known, for example, which are still able to grow on
10   glucose despite having a mutation in the transaldolase gene (Schaaff L. et al., Eur. J. Biochem. 188 (1990) 597-603). Accordingly, a transaldolase should not exert any essential influence on cell growth. This is supported by the fact that bacterial species are also known which are
15   able to grow in the absence of a transaldolase (Feldmann S.D. et al., Appl. Microbiol. Biotechnol. 38 (1992) 354-62).

In addition, hexoses such as glucose, in contrast to xylose and other pentoses, can also be
20   metabolized through degradation pathways other than the pentose phosphate pathway. It is therefore in no way self-evident that transaldolase has an essential function in glucose degradation.

It may also be noted that an increased flow by
25   way of the enzymes of the pentose phosphate pathway was measured in vitro in extracts of *Saccharomyces cerevisiae* cells in which the activity of a transaldolase was increased (Senac T. et al., Appl. Environ. Microbiol. 57 (1991) 1701-6). However, the experimental conditions which
30   were selected in this study do not permit any transfer of the results to the natural phenomena of metabolic physiology which occur in living microorganisms, in particular bacteria. In addition, it may be noted that Liao J.C. et al. (Biotechn. Bioeng. 52 (1996) 129-140 have

shown that when the activities of a transaldolase and AroG
are simultaneously increased, DAHP formation increases.
This effect is to be ascribed mainly to the increase in
AroG activity. An increase in the production of substances
5   as a result of an increase in transaldolase activity as
such is not disclosed or indicated. It is therefore in no
way to be expected that there is a causal relationship
between an increase in transaldolase activity in
microorganisms and the provision of Ery4P for the improved
10   production of substances. Increasing the activity
(overexpression) of a transaldolase in accordance with the
present invention makes available an alternative process
for implementing an increased flow of carbon through the
pentose phosphate pathway and consequently effecting an
15   improved provision of the primary metabolite Ery4P. An
increased quantity of Ery4P is consequently available for
the microbial synthesis of substances in whose synthesis
at least one intermediate of the pentose phosphate
pathway, and in particular Ery4P, is involved. The
20   inventors have shown that the improved availability of
Ery4P occurs both in microorganisms in which the activity
of a PEP-dependent sugar-uptake system is present or
reduced and in microorganisms in which such an activity is
absent. Thus, according to the invention not only
25   organisms which still have active phosphotransferase
systems (PTS) or phosphotransferase systems with reduced
activity (pts· strains) may be used but also organisms in
which the PTS has been switched off (pts⁻ strains).
        Within the meaning of the invention, substances
30   are to be understood as being, for example, fine chemicals
such as aromatic amino acids, indigo, indoleacetic acid,
adipic acid, melanin, quinones and benzoic acid, and also
their potential derivatives and secondary products - or,
in a general manner, secondary products of intermediates

of the pentose phosphate pathway. However, this should
also include all the compounds, for example pyridoxine and
its derivatives, whose biochemical synthesis is promoted
by the provision of erythrose 4-phosphate. Within the
5   context of this invention, all these substances are also
regarded as being substances from aromatic metabolism. It
may be noted in this context that further genetic
alterations to the substance-producing microorganisms are
required, in addition to the novel interventions, for
10   preparing indigo, adipic acid and other unnatural
secondary products. The process according to the invention
is particularly suitable for the preparation of substances
in microorganisms which have not, prior to the increasing
of the transaldolase activity, been selected as strains
15   with a switched-off phosphotransferase system (pts⁻
strains) from strains which previously harboured a
phosphotransferase system (pts⁺ strains). In this
connection it may be noted that the non-prepublished
patent specification WO-96/34961 describes such a
20   selection from what were previously pts⁺ strains, but in
this study this selection precedes the increasing of the
transketolase or transaldolase activity.

          With regard to increasing the activity of a
transaldolase, preference is given to increasing the
25   activity of an *Escherichia coli* transaldolase and, in
particular, the activity of *Escherichia coli* transaldolase
B (TalB). When the corresponding talB gene is used, this
gene preferably originates from *Escherichia coli* K12 or
from a strain which is derived therefrom. Besides this,
30   however, any gene is also suitable whose gene product
catalyses a reaction which corresponds to that of
transaldolase, that is the conversion of sedoheptulose
7-phosphate plus glyceraldehyde 3-phosphate to Ery4P and
fructose-6-phosphate.

In a particularly advantageous embodiment of the
invention, the activity of a transketolase is increased in
addition to increasing the activity of a transaldolase. In
the presence of pentoses, the expression of a

5   transketolase gene on its own leads to a build up of
metabolites in the pentose phosphate pathway
(Feldmann S.D., Appl. Microbiol. Biotechnol. 38 (1992)
354-61). This has a harmful effect on the cell and
results, inter alia, in the transformed cells growing at a

10   diminished rate. The inventors also observed an identical
effect in the case of an *Escherichia coli* strain which was
growing on glucose and which exhibited increased
transketolase activity. However, simultaneously increasing
the activity of a transketolase as well as that of a

15   transaldolase has now been found to be very beneficial for
providing Ery4P and does not exhibit the negative side
effects of a sole overexpression of a transketolase. The
accumulation of metabolites of the pentose phosphate
pathway which occurs when the activity of a transketolase

20   is increased consequently fails to materialize as a result
of increasing the activity of a transaldolase, as the
result of which the impairment in the growth of the cells
is not only halted but even converted into an increase in
growth. It turns out, therefore, that increasing the

25   activity of a transketolase particularly in strains having
an increased activity of transaldolase in accordance with
the invention is a sensible measure.

        With regard to increasing the activity of a
transketolase, preference is given to increasing the

30   activity of an *Escherichia coli* transketolase and, in
particular, the activity of *Escherichia coli* transketolase
A (TktA). When the corresponding tktA gene is used, this
gene preferably originates from *Escherichia coli* K12 or
from a strain which is derived therefrom. In addition to

this, however, any gene is also suitable whose gene
product catalyses a reaction which corresponds to that of
transketolase, that is the conversion of ribose
5-phosphate plus xylulose 5-phosphate to sedoheptulose

5   7-phosphate plus glyceraldehyde 3-phosphate or the
conversion of xylulose 5-phosphate plus Ery4P to fructose
6-phosphate plus glyceraldehyde 3-phosphate.

        In another particularly preferred embodiment of
the invention, the activity of a transport protein for the

10   PEP-independent uptake of a sugar and/or the activity of a
kinase which phosphorylates the corresponding sugar is/are
increased in addition to increasing the activity of a
transaldolase or increasing the activity of a
transaldolase and a transketolase. In the case of the

15   transport protein, the activity of this protein is
understood as being the protein-mediated uptake rate. The
additional increase in the activity of one of these latter
proteins, i.e. the transport protein and the kinase, or of
both proteins makes it possible to achieve an even higher

20   production of substances, in particular aromatic amino
acids, due to the fact that PEP is provided in increased
quantity for the condensation with Ery4P to form the
primary metabolite of the general pathway for
biosynthesizing aromatic compounds, i.e. deoxy-D-

25   arabinoheptulosonate-7-phosphate (DAHP).

        It is advisable to use a facilitator as the
transport protein for the PEP-independent uptake of a
sugar, that is a transport protein which acts in accor-
dance with the principle of protein-mediated facilitated

30   diffusion. In particular, it is suitable to use the
glucose facilitator protein (Glf) from *Zymomonas mobilis*.
When the latter is used, the protein-encoding gene, *glf*,
is obtained, for example, from *Z. mobilis* ATCC 10988, ATCC
29191 or ATCC 31821. However, other bacterial sugar

transport genes whose gene products transport glucose, fructose or sucrose, for example, and in doing so do not use any PEP, for example the GalP system from *Escherichia coli*, are also suitable for the novel process. Genes for

5   sugar transport systems, such as HXT1 to HXT7, from eukaryotic microorganisms, such as *Saccharomyces cerevisiae*, *Pichia stipitis* or *Kluyveromyces lactis*, or sugar transport genes from other organisms in general, can also be used, provided that they can be expressed in a

10   functional manner in the microorganisms and that the gene products can, in this context, operate without using PEP for transporting the sugars. It is particularly advisable for it to be possible to express the sugar transport genes in amino acid-producing microorganisms (amino acid

15   producers).

It is particularly suitable, therefore, to use the facilitator gene *glf*, isolated from *Z. mobilis* ATCC 31821, for taking up sugars such as glucose, fructose or mannose when preparing aromatic amino acids in accordance

20   with the novel process. (Parker C. et al., Mol. Microbiol. 15 (1995) 795-802; Weisser P. et al., J. Bacteriol. 177 (1995) 3351-4). The process according to the invention is particularly suitable for the preparation of aromatic amino acids in microorganisms in which the PTS system has

25   a reduced activity or is completely absent. In such cases the PTS system may be reduced or switched off before but also after the increasing of the transaldolase activity.

The *glf* gene, in particular, should preferably be inserted at a low gene copy number in order to avoid

30   harmful effects on the cell due to excessive expression of membrane proteins. Thus, a gene copy number of from 2 to 5 is preferred, for example, for the *glf* gene. It is particularly advantageous to insert the *glf* gene into one of the genes of the *pts*HI-crr operon, for example into the

ptsI gene.

When a sugar-phosphorylating kinase is being
employed, it is advisable to use a hexose-phosphorylating
kinase, preferably a kinase, in particular glucokinase
5   (Glk), from *Zymomonas mobilis*. When the latter is used,
the protein-encoding gene, *glk*, is derived, for example,
from *Z. mobilis* ATCC 10988, ATCC 29191 or ATCC 31821.
Other genes for hexose-phosphorylating kinases from
bacteria whose gene products phosphorylate hexoses while
10 consuming ATP, such as a fructokinase or a mannokinase,
are likewise suitable for the novel process. Furthermore,
genes for, for example, kinases from eukaryotic micro-
organisms, such as *Saccharomyces cerevisiae*, or, in a
general manner, genes for sugar-phosphorylating kinases
15 from other organisms, are also suitable, provided they can
be expressed in a functional manner in the microorganisms,
in particular amino acid producers, and can operate
without using PEP to phosphorylate the sugars.
The glucokinase gene, *glk*, for phosphorylating glucose,
20 which gene is isolated from *Z. mobilis* ATCC 29191, is
particularly suitable for preparing aromatic amino acids
in accordance with the novel process.

The availability of PEP for producing the first
intermediate of aromatic amino acid metabolism can be
25 limited in microorganisms in which the flow of material
towards Ery4P is increased. In these cases, it can be
advantageous to reduce, or completely switch off, other
PEP-consuming reactions in the metabolism, such as the
reaction of the PEP:sugar phosphotransferase system (PTS),
30 which catalyses a PEP-dependent sugar uptake, if this
system is present. According to the invention, use can be
made both of organisms which still possess active PTS
genes (*pts⁺* strains) and, for the further improvement of
the process, of *pts* mutants in which the activity of the

pts is decreased (also to be considered as *pts*ˈ in the
present case) or in which the *pts* is switched off (*pts*⁻
strains). A decrease of this nature can either be effected
at the enzymic level or by using genetic methods, for
5   example by inserting a *glf* and/or a *glk* gene into the
chromosome and, in particular, into the locus of the *pts*I
gene, a procedure which simultaneously stabilizes the
recombinant DNA in the chromosome (segregation stability)
and consequently means that the use of a vector can be
10   dispensed with. The inventors' experiences have shown that
reducing or switching off the activity of the PTS should
preferably not take place before the activity of the
transaldolase is increased.

            Within the meaning of the invention, measures
15   for increasing the activity are to be understood as being
all measures which are suitable for increasing the
activity of the transaldolase, of the transketolase, of
the transport protein, and of the kinase. The following
measures are particularly suitable for this purpose:
20   - introduction of genes, for example using vectors or
temperate phages;
- increasing the gene copy number, for example using
plasmids with the aim of introducing the genes according
to the invention into the microorganism at an increased
25   copy number, that is at a copy number which is slightly
(e.g. from 2 to 5 times) increased to highly (e.g. from 15
to 50 times) increased;
- increasing gene expression, for example by increasing
the rate of transcription, for example by using promoter
30   elements such as P*tac*, P*tet* or other regulatory nucleotide
sequences, and/or by increasing the rate of translation,
for example by using a consensus ribosome binding site;
- increasing the endogenous activity of enzymes which are
present, for example by means of mutations which are

produced in a random manner by conventional methods, for example using UV irradiation or mutation-eliciting chemicals, or by means of mutations, such as deletion(s), insertion(s) and/or nucleotide exchange(s), which are

5   produced in a specific manner using genetic engineering methods;
- increasing the activity of enzymes by altering the structure of enzymes, for example by means of mutagenesis using physical, chemical or molecular biological or other

10   microbiological methods;
- using deregulated enzymes, for example enzymes which are no longer subject to feedback inhibition;
- introduction of corresponding genes which encode the deregulated enzymes.

15   Combinations of the abovementioned methods and other, analogous methods can also be employed for increasing the activity. The endogenous or introduced activity of transport proteins can be increased, for example, by cloning the gene using the abovementioned

20   methods, for example, or by selecting mutants which exhibit an increased transport of substrates.

            Preferably, the activity is increased by integrating the gene, or the genes, into a gene structure, or into several gene structures, with each gene or the genes

25   being introduced into the gene structure as (a) single copy(ies) or at an increased copy number.
Within the meaning of the invention, a gene structure is to be understood as being a gene and any other nucleotide sequence which carries the genes according to the

30   invention. Appropriate nucleotide sequences can, for example, be plasmids, vectors, chromosomes, phages or other nucleotide sequences which are not closed circularly.

            A chromosome within the meaning of the invention

is a chromosome into which at least one gene according to
the invention has been inserted, with the resulting
nucleic acid sequence containing at least one gene, or one
gene copy, more than is naturally contained in this
5   chromosome. On the other hand, for example, homologous
recombination within a gene locus leads to a chromosome
which does not necessarily have to differ from the natural
form. The chromosomes which are prepared by homologous
recombination are not, therefore, to be regarded as being
10   in accordance with the invention if the natural number of
homologous genes is not exceeded.

Within the meaning of the invention, a gene
structure is also to be understood as being a combination
of the abovementioned gene carriers, such as vectors,
15   chromosomes and temperate phages, on which the genes
according to the invention are distributed. For example,
two *tal* genes can be introduced into the cell on a vector
or two *tal* genes can be inserted into a chromosome. In
addition, a further gene can, for example, be introduced
20   into the cell using a phage. The same applies to the other
genes according to the invention. These examples are not
intended to exclude other combinations of gene
distributions from the invention. In any case, it is
crucial that the number of genes contained in the
25   microorganism according to the invention exceeds the
natural number of the corresponding genes. Preferably, the
number of *glf* genes, for example, will be increased by a
factor of from 2 to 5 in order to achieve the increase in
activity according to the invention. No cell-toxic effect
30   will appear at these concentrations. However, it is also
conceivable to introduce the genes according to the
invention into the microorganism at a higher copy number
of up to 50 gene copies of a form having the same effect.

In the novel process for producing substances,

preference is given to employing microorganisms in which
one or more enzymes which are additionally involved in
synthesizing the substances are deregulated and/or have an
increased activity.

5   These enzymes are, in particular, the enzymes of
aromatic amino acid metabolism and, especially, DAHP
synthase, shikimate kinase and chorismate
mutase/prephenate dehydratase, and also all the other
enzymes which are involved in synthesizing intermediates

10   of aromatic metabolism and their secondary products.
            Apart from the enzymes according to the
invention, the deregulation and overexpression of DAHP
synthase in particular is of importance for preparing
substances such as adipic acid, bile acid and quinone

15   compounds and their derivatives. In addition, shikimate
kinase should be deregulated, and its activity increased,
in order to achieve superelevated synthesis of, for
example, tryptophan, tyrosine, indigo and derivatives of
hydroxybenzoic acid and aminobenzoic acid and naphtho-

20   quinones and anthroquinones and also their secondary
products. Deregulated and overexpressed chorismate
mutase/prephenate dehydratase is additionally of par-
ticular importance for efficiently producing phenylalanine
and phenylpyruvic acid and their derivatives. However,

25   this is also intended to encompass all the other enzymes
whose activities contribute to the biochemical synthesis
of substances, that is compounds whose production is
promoted by the provision of erythrose 4-phosphate, for
example pyridoxine and its derivatives. It may be noted

30   that further genetic alterations to the substance-
producing microorganisms, in addition to the novel
interventions, are required for the purpose of preparing
indigo, adipic acid and other unnatural secondary
products.

The novel process is suitable, in particular,
for preparing aromatic amino acids, in particular phenyl-
alanine. In the latter case, the gene expression and/or
the enzyme activity of a deregulated DAHP synthase (e.g.
5   in *E. coli* AroF or AroH) and/or of a likewise deregulated
chorismate mutase/prephenate dehydratase (PheA) is
preferably increased.

     Escherichia species, and also microorganisms of
the genera *Serratia*, *Bacillus*, *Corynebacterium* or *Brevi-*
10   *bacterium*, and other strains which are known from conven-
tional amino acid methods, are suitable for use as
production organisms. *Escherichia coli* is particularly
suitable.

     Another object of the invention is to provide
15   suitable gene structures, and transformed cells carrying
these gene structures, which enable the process to be
implemented in a particularly successful manner.
Within the context of the invention, novel gene structures
are now made available, which gene structures contain, in
20   recombinant form, a) a gene encoding a transaldolase or a
gene encoding a transaldolase and a gene encoding a
transketolase, and b) at least one gene encoding a
transport protein for the PEP-independent uptake of a
sugar or encoding a kinase which phosphorylates a sugar.
25   In these gene structures, it is preferred that the gene
for the transport protein encode a facilitator and the
gene for the kinase encode a hexose-phosphorylating
kinase. In particular, the genes for the transaldolase and
the transketolase are derived from *Escherichia coli* and
30   the genes for the transport protein and the kinase are
derived from *Zymomonas mobilis*.

     Gene structures are particularly advantageous in
which the gene for the transaldolase is *Escherichia coli*
talB and the gene for the transketolase is *Escherichia*

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coli tktA, while the gene for the transport protein is
Zymomonas mobilis glf and the gene for the kinase is
Zymomonas mobilis glk.

The appropriate genes are isolated, and the
5   cells are transformed, in accordance with current methods:
the complete nucleotide sequences of the *talB* and *tktA*
genes from *Escherichia coli* K12 are known (Yura T. et al.,
Nucl. Acid Res. 20 (1992) 3305-8; Sprenger G.A., Biochim.
Biophys. Acta 1216 (1993) 307-10; Sprenger G.A. et al., J.
10   Bacteriol. 177 (1995) 5930-9) and deposited in databases
such as that of the EMBL in Heidelberg. When the
Escherichia coli talB gene is being cloned, the polymerase
chain reaction (PCR) method is suitable, for example, for
specifically amplifying the gene using chromosomal DNA
15   from *Escherichia coli* K12 strains (Sprenger G.A. et al.,
J. Bacteriol. 177 (1995) 5930-9). The homologous
complementation of a transketolase-deficient mutant is
suitable, for example, when cloning the *Escherichia coli
tktA* gene (Sprenger G.A. in: Bisswanger H. et al.,
20   Biochemistry and physiology of thiamine diphosphate
enzymes, VCH (1991) 322-6).

When cloning the *Zymomonas mobilis* transport
gene *glf* or the *Zymomonas mobilis* glucokinase gene *glk*,
for example, PCR, for example, is suitable for
25   specifically amplifying the gene using chromosomal DNA
from *Zymomonas mobilis* strains ATCC 29191 or ATCC 31821,
as is also the heterologous complementation of *Escherichia
coli* mutants which are defective in PTS functions and
which are therefore unable to transport glucose, for
30   example (Snoep J.L. et al., J. Bacteriol. 174 (1994) 1707-
8; Parker C. et al., Mol. Microbiol. 15 (1995) 795-82;
Weisser P. et al., J. Bacteriol. 177 (1995) 3351-4). After
isolating the genes and recombining them in vitro with
known low copy number vectors such as pACYC184, pACYC177,

pSC101 or pZY507 (Weisser P. et al., J. Bacteriol. 177
(1995) 3351-4), the host cell is transformed using
chemical methods, electroporation, conjugation or
transduction.

5   The isolated transaldolase gene can be
integrated, together with one or more of the genes
described within the context of the invention, in any
combination, into a gene structure or into several gene
structures. Without considering the precise allocation to
10   gene structures, this leads to combinations such as *talB*,
talB + *tktA*, *talB* + *glf*, *talB* + *glk*, *talB* + *glf* + *glk*,
talB + *tktA* + *glf*, *talB* + *tktA* + *glk* or *talB* + *tktA* +
glf + *glk*.

          Gene structures are advantageous which contain
15   at least one regulatory gene sequence which is assigned to
one of the genes. Thus, reinforcement of regulatory
elements can preferably be effected at the level of
transcription by, in particular, reinforcing the
transcription signals. This can be effected, for example,
20   by increasing the activity of the promoter or the
promoters by altering the promoter sequences which are
located upstream of the structural genes or by completely
replacing the promoters with more effective promoters.
Transcription can also be reinforced by exerting an
25   appropriate influence on a regulatory gene which is
assigned to the genes; in addition to this, however, it is
also possible to reinforce translation by, for example,
improving the stability of the messenger RNA (mRNA).

          The most suitable gene structures are those in
30   which at least one of the described genes is incorporated
such that it is under the control of an inducible
promoter. When the genes are arranged on a gene structure
according to the invention, a promoter can be located
upstream of a gene or be located, as a common promoter,

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upstream of several genes, or use can be made of two
opposed promoters between which the genes are arranged
such that they are read off in opposite directions. In
this context, the *glf* gene, for example, can be located

5   downstream of a relatively weak promoter (e.g. P*tet*) and
other genes can be under the control of the *tac* promoter.
One or more DNA sequences can be located upstream and/or
downstream of the genes contained in a gene structure,
with or without an upstream promoter or with or without an

10 assigned regulatory gene. By means of using inducible
promoter elements, e.g. *lacI*^{q}/P*tac*, it is possible to
switch on new functions (induction of enzyme synthesis),
for example by adding chemical inducers such as iso-
propylthiogalactoside (IPTG).

15   The object of the invention is also achieved by
providing transformed cells which harbour a gene structure
according to the invention in replicable form.
            Within the meaning of the invention, a
transformed cell is to be understood as being any

20 microorganism which carries a gene structure according to
the invention, which gene structure brings about the
increased formation of substances in the cell. The host
cells can be transformed by means of chemical methods
(Hanahan D., J. Mol. Biol. 166 (1983) 557-580) and also by

25 means of electroporation, conjugation or transduction.
            For the transformation, it is advantageous to
employ host cells in which one or more enzymes which are
additionally involved in the synthesis of the substances
are deregulated and/or have an increased activity. A

30 microorganism strain, in particular *Escherichia coli*,
which is producing an aromatic amino acid or another
substance according to the invention is transformed with
the gene structure which contains the relevant genes. For
transforming with the gene structures, it is advantageous

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to employ host cells in which the activity of the PEP-
dependent sugar uptake system, if present, is decreased or
switched off.

In particular, transformed cells are provided
5   which are able to produce an aromatic amino acid, with the
aromatic amino acid preferably being L-phenylalanine.

Using the novel process, and the microorganism
which has been transformed in accordance with the teaching
of the invention, a broad spectrum of substrates can be
10   employed for producing substances. Within the context of
the invention, a process for the microbial preparation of
substances is consequently also provided in which cells
which have been transformed in accordance with the
invention and in which a gene structure is present which
15   contains at least one regulatory gene sequence which is
assigned to one of the genes are cultured, with enzyme
synthesis being induced in the microorganisms after at
least 2 cell divisions (beginning of the exponential
growth phase). Consequently, the production of the micro-
20   organisms can be increased independently of their growth.

In a particularly preferred embodiment of the
novel process, transformed cells are employed which, in
addition to the intermediates of the pentose phosphate
pathway, also contain an increased availability of other
25   metabolites of central metabolism. These metabolites
include, for example, pyruvate from glycolysis or
gluconeogenesis, or oxaloacetate from the citric acid
cycle. Furthermore, the relevant compound, or its pre-
cursors, that is the precursors of pyruvate or of
30   metabolites of the citric acid cycle, such as fumarate or
malate, can be made available to the growing cells by
feeding.

The invention is explained in more detail below
with the aid of a few implementation examples. The

following strains have been deposited at DSM under the
terms of the Budapest treaty:

DSM 11210 *Escherichia coli* AT2471/pZYTT7tal

5   DSM 11209 *Escherichia coli* AT2471/pZY557tkttal

DSM 11206 *Escherichia coli* AT2471GP704glfint PTS⁺

DSM 11205 *Escherichia coli* AT2471glfint PTS⁻

The host organism employed, i.e. AT2471, has
10   been deposited by Taylor and Trotter (Bacteriol. Rev. 13
(1967) 332-353) in the CGSC under number 4510 and is
freely accessible.

The characteristics and the provenance of all
the microorganisms employed within the context of this
15   patent application are described in detail in Table 1.

The text which follows is intended to indicate
the materials and methods employed and to support the
invention with experimental examples and comparative
examples:

20

General methods

In the genetic studies, strains of *E. coli* were,
unless otherwise indicated, cultured on LB medium
consisting of Difco bacto-tryptone (10 g·l⁻¹), Difco yeast
25   extract (5 g·l⁻¹) and NaCl (10 g·l⁻¹). Depending on the
resistance properties of the strains employed, carbeni-
cillin (20-100 mg·l⁻¹) and/or chloramphenicol (17-34 mg·l⁻
¹) was/were added to the medium if necessary. For this,
carbenicillin was first of all dissolved in water, and
30   chloramphenicol in ethanol, and the solutions were added,
after having been sterilized by filtration, to the
previously autoclaved medium. Difco bacto-agar (1.5%) was
added to the LB medium for preparing agar plates.
Plasmid DNA was isolated from *E. coli* by means of alkaline

lysis using a commercially available system (Quiagen,
Hilden). Chromosomal DNA was isolated from *E. coli* and *Z.
mobilis* as described by Chen and Kuo (Nucl. Acid Res. 21
(1993) 2260).

5   Restriction enzymes, DNA polymerase I, alkaline
phosphatase, RNase and T4 DNA ligase were used in accor-
dance with the producers' instructions (Boehringer,
Mannheim, Germany or Promega, Heidelberg, Germany). For
restriction analysis, the DNA fragments were fractionated
10 in agarose gels (0.8%) and isolated from the agarose by
means of extraction using a commercially available system
(Jetsorb Genomed, Bad Oeynhausen, Germany).

For Southern analyses, chromosomal DNA (10 μg)
was digested with restriction enzymes, size-fractionated
15 by gel electrophoresis and transferred to a nylon membrane
(Nytran 13, Schleicher and Schuell, Dassel, Germany) by
means of vacuum-mediated diffusion (VacuGene System,
Pharmacia, Freiburg, Germany). Appropriate DNA fragments
were isolated, labelled with digoxigenin-dUTP and used as
20 probes. Labelling, hybridization, washing procedures and
detection were performed with the aid of a commercially
available labelling and detection system (Boehringer,
Mannheim, Germany).

For transformation, the cells were incubated at
25 37°C and 200 rpm for 2.5-3 h in LB medium (5 ml tubes). At
an optical density (620 nm) of approx. 0.4, the cells were
centrifuged down and taken up in one tenth the volume of
TSS (LB medium containing 10% (w/v) PEG 8000, 5% (v/v)
DMSO and 50 mM $MgCl_2$). After a 30-minute incubation at 4°C
30 with from 0.1 to 100 ng of DNA, and subsequent incubation
at 37°C for 1 h, the cells were plated out on LB medium
containing an appropriate antibiotic.

Example 1

<u>Preparation of pZY557tal, pBM20tal and pZY557tkttal as
prototypes of plasmid-based gene structures according to
the invention, and of pZY557tkt</u>

Plasmid pZY507 (Weisser et al. 1995 J. Bacteriol

5   177: 3351-3345) was opened using the restriction enzymes
*Bam*HI and *Hind*III and the larger fragment (10.1 kB of DNA)
was isolated. A part of the multiple cloning site was
excised from vector pUCBM20 (Boehringer, Mannheim,
Germany) using *Bam*HI and *Hind*III and ligated to the large

10   pZY507*Bam*HI/*Hind*III fragment, resulting in vector pZY557,
which is similar to vector pZY507 apart from the other
restriction cleavage sites. The *tal*B gene was amplified
from vector pGSJ451 (Sprenger G.A. et al., J. Bacteriol.
177 (1995) 5930-36) by means of PCR. For this, use was

15   made of oligonucleotide I:
5' CCGCAT<u>GCTGTTTAAAGAGAAATA</u> 3' (the base pairs which are
underlined here correspond to base pairs 84 to 101 of the
*tal*B sequence from Sprenger G.A. et al., J. Bacteriol. 177
(1995) 5930-6), which was provided with a cleavage site

20   for the restriction enzyme *Sph*I, and of the commercially
obtainable M13/pUC sequencing primer (No. 1010093,
Boehringer Mannheim, Germany) as oligonucleotide II. The
resulting amplified DNA fragment contains a cleavage site
for *Sph*I at each end and was cleaved with the restriction

25   enzyme *Sph*I. This fragment was then ligated to vectors
pZY557 and pUCBM20, respectively, which vectors had also
been linearized with *Sph*I. The recombinant plasmids
pZY557tal and pBM20tal, respectively, were obtained after
transforming *E. coli* with the two ligation solutions and

30   cloning the transformants. The *tkt*A gene was amplified by
PCR from the vector pGSJ427 (Sprenger G.A. et al., Eur. J.
Biochem. 230 (1995) 525-32) with a cleavage site for the
restriction enzyme *Not*I being inserted at the 5' end and a
cleavage site for the enzyme *Sph*I being inserted at the 3'

end. Use was made in this case of oligonucleotides III:
5'TTAGCGGCCGCCCTTCATCATCCGATCT 3' (the base pairs which
are underlined here correspond to base pairs 126 to 146 of
the tktA sequence from Sprenger G.A., Biochim. Biophys.
5 Acta. 1216 (1993) 307-10), which was provided with a
cleavage site for the restriction enzyme NotI, and IV: 5'
ATAGCATGCTAATTACAGCAGTTC 3' (the base pairs which are
underlined here correspond to base pairs 2018 to 2036 of
the tktA sequence from Sprenger G.A., Biochim. Biophys.
10 Acta. 1216 (1993) 307-10), which was provided with a
cleavage site for SphI. The resulting PCR fragment was
cleaved with NotI and SphI and ligated into vector
pZY557tkt, which had been treated in the same way,
resulting in vector pZY557tkt. pZY557tkt was then opened
15 with SphI and ligated to the SphI-cleaved PCR fragment
containing talB. After transforming E. coli and cloning
the transformants (see above), plasmids were obtained
which contained the tktA gene and the talB gene oriented
in the same direction downstream of the tac promoter and
20 which were subsequently used as gene structure
pZY557tkttal.

        The resulting transformants were stored at -80°C
on LB medium in the form of glycerol cultures (30%). When
needed, the glycerol cultures were thawed directly before
25 use.

Example 2
Effect of the increased activity of a transaldolase on the
growth of strains in which transketolase activity is also
30 increased

        The growth of the E. coli strains AT2471,
AT2471/pZY557tkt, AT2471/pZT557tal and AT2471/pZY557tkttal
on glucose was investigated in mineral medium. This con-
sisted of Na citrate·3H$_2$O (1.0 g·l^{-1}), MgSO$_4$·7H$_2$O (0.3 g·l^{-}

^{1}), KH_2PO_4 (3.0 g$\cdot$l^{-1}), K_2HPO_4 (12.0 g$\cdot$l^{-1}), NaCl (0.1 g$\cdot$l^{-1}), $(NH_4)_2SO_4$ (5.0 g$\cdot$l^{-1}), $CaCl_2\cdot2H_2O$ (15.0 mg$\cdot$l^{-1}), $FeSO_4\cdot7H_2O$ (0.75 g$\cdot$l^{-1}) and L-tyrosine (0.04 g$\cdot$l^{-1}). Additional minerals were added in the form of a trace element

5   solution (1 ml$\cdot$l^{-1}), which was composed of $Al_2(SO_4)_3\cdot18H_2O$ (2.0 g$\cdot$l^{-1}), $CoSO_4\cdot6H_2O$ (0.7 g$\cdot$l^{-1}), $CuSO_4\cdot5H_2O$ (2.5 g$\cdot$l^{-1}), H_3BO_3 (0.5 g$\cdot$l^{-1}), $MnCl_2\cdot4H_2O$ (20.0 g$\cdot$l^{-1}), $Na_2MoO_4\cdot2H_2O$ (3.0 g$\cdot$l^{-1}), $NiSO_4\cdot3H_2O$ (2.0 g$\cdot$l^{-1}) and $ZnSO_4\cdot7H_2O$ (15.0 g$\cdot$l^{-1}). Vitamin B1 (5.0 mg$\cdot$l^{-1}) was dissolved in

10 water and added, after having been sterilized by filtration, to the medium after the latter had been autoclaved, as were carbenicillin and/or carbenicillin and chloramphenicol as the need arose. Glucose (30 g$\cdot$l^{-1}) was autoclaved separately and likewise added to the medium

15 after the latter had been autoclaved.

    For the experiments, shaking flasks (1000 ml containing 100 ml of mineral medium) were inoculated with 2 ml of glycerol culture and incubated at 37°C and 150 rpm for 72 h on an orbital shaker. After reaching an optical

20 density (620 nm) of ≈ 1, the cells were induced by adding 15-100 μM IPTG. The optical density of the culture was measured at regular intervals up to this point. Under the above-described conditions, the host organism *E. coli* AT2471 reached an optical density of 1.2 after 7.25 h. The

25 increase in the activity of the transketolase in mutant AT2471/pZY557tkt led to a marked reduction in the rate of growth; while having an identical optical density at the beginning of the experiment, this strain only reached an optical density of 0.49 after 7.25 h. The growth-

30 inhibiting effect is probably attributable to the synthesis of inhibitory concentrations of metabolic intermediates of the pentose phosphate pathway.

    The increase in the activity of the transaldolase in mutant AT2471/pZY557tal also led to a

marked reduction (almost as marked as in the case of the
transketolase) in the growth rate; an optical density of
0.52 was reached after 7.25 h. It was possible to reach an
optical density of 1.1 after 7.25 h by increasing the

5   activity of the transaldolase in addition to that of the
transketolase, as was effected in *E. coli*
AT2471/pZY557tkttal. This result makes it clear that
additionally increasing the activity of a transaldolase in
a strain having an increased activity of transketolase

10   cancels the negative effects of only increasing the
activity of the transketolase and permits virtually
uninhibited growth.

        Example 3

15   Determination of the enzyme activities of the
transaldolase

                In order to determine the activity of the trans-
aldolase, the cells of *E. coli* AT2471, AT2471/pZY507,
AT2471/pZY557tal and AT2471/pZY557tkttal were cultured as

20   described above with, however, 3-(morpholino)propane-
sulphonic acid (MOPS, 16.7 $g \cdot l^{-1}$) being added to the
medium and the phosphate concentration being lowered to
one tenth of the concentration which was employed in the
growth experiments. In order to check the induction of the

25   cells, parallel experimental mixtures were set up, one of
which mixtures was induced by adding IPTG (20 µM) after 7
cell divisions (OD ≈ 1). 20 ml of culture broth were
removed from all the mixtures directly before adding the
inducer to the relevant flasks and 3 h after the time of

30   induction and the cells were sedimented at 6000 g for
10 min at 4°C.

                The harvested cells were washed in 50 mM
glycylglycine buffer, pH 8.5, containing 1 mM
dithiothreitol, 10 mM $MgCl_2$ and 0.5 mM thiamine

diphosphate. The cells in the sediment were disrupt d by
ultrasonic treatment (Branson 250 Sonifier fitted with a
microtip) in a sonication cycle of 25% and at an intensity
of 40 watts for 4 min per ml of cell suspension. After
5   centrifuging at 18,000 g for 30 min at 4°C, the
supernatant (crude extract) was used for measuring the
activity of the transaldolase.

The transaldolase activity in the crude extract
was determined using an enzymic test which was optically
10   coupled to the formation of NAD. For this, the crude
extract was incubated in a total volume of 1 ml containing
0.8 mM fructose 6-phosphate, 4 mM erythrose 4-phosphate
and 0.3 mM NADH in buffer (50 mM glycylglycine, pH 8.5,
1 mM dithiothreitol, 10 mM $MgCl_2$ and 0.5 mM thiamine
15   diphosphate). The resulting glyceraldehyde 3-phosphate was
reacted with the enzymes triosephosphate isomerase (9 U)
and glycerol 3-phosphate dehydrogenase (3 U), which
enzymes were also added, with the formation of NAD. The
oxidation of NADH was monitored spectrophotometrically at
20   340 nm (ε = $6.3 \cdot 10^3$ $1 \cdot mol^{-1} \cdot cm^{-1}$), with the conversion of
1 μmol of NADH being equivalent to the consumption of
1 μmol of fructose 6-phosphate. The turnover of 1 μmol of
NADH per min was defined as 1 U.

The protein concentration in crude extracts was
25   determined as described by Bradford (Anal. Biochem. 72
(1976) 248-254) using a commercially available colour
reagent. Bovine serum albumin was used as the standard.

Table 2 shows the results of the enzyme
measurements when using the host strain E. coli AT2471 and
30   its mutants which harbour one of the plasmids pZY557,
pZY557tal or pZY557tkttal. At the time of induction, the
host strain and the strain carrying the vector pZY557 were
found to have a transketolase activity of about 0.65 U·(mg
of protein)$^{-1}$. As shown for E. coli AT2471/pZY557, this

value increased in three hours, as the result of physio-
logical growth, to 0.8 U·(mg of protein)$^{-1}$. Due to the
absence of the *tal* gene on the vector employed, it was not
to be expected that the induction which was performed in
5 this experiment would have any effect.

In parallel experimental mixtures, *E. coli*
AT2471/pZY557tal was found to have transketolase
activities of 0.53 and 0.61 U·(mg of protein)$^{-1}$,
respectively, at the time of induction. While the value
10 for the transaldolase activity increased in 3 h to only
0.6 U·(mg of protein)$^{-1}$ when the culture was not induced,
the transaldolase activity achieved a value of 1.06 U·(mg
of protein)$^{-1}$ as a result of induction. In other
experimental mixtures, *E. coli* AT2471/pZY557tkttal was
15 used in what were otherwise identical experiments; in this
case, the transaldolase activity had increased in the
first 3 h after inducing the cells from 0.8 to 1.16 U·(mg
of protein)$^{-1}$, which corresponded to a doubling of the
activity as compared with the corresponding experiments
20 using the host strain.

Example 4
Production of substances using strains which exhibit an
increased transketolase activity in addition to the
25 increased transaldolase activity

The medium which was employed for the growth
experiments was used for determining synthetic perform-
ance. The cultures of *E. coli* AT2471 and
AT2471/pZY557tkttal were induced at an optical density of
30 1 and the period of culture was extended to 72 h. After 24
and 48 h, the pH of the cultures was measured and brought
back to the starting value of 7.2, if required, by adding
KOH (45%). In addition, samples (2 ml) were taken after
24, 48 and 72 h for determining the optical density as

well as the concentrations of glucose and L-phenylalanine.

The phenylalanine concentration was determined
by means of high pressure liquid chromatography (HPLC,
Hewlett Packard, Munich, Germany) in combination with
5   fluorescence detection (extinction 335 nm, emission
570 nm). A Nucleosil-120-8-C18 column (250 · 4.6 mm) was
used as the solid phase; the column was eluted using a
gradient (eluent A: 90% 50 mM phosphoric acid, 10%
methanol, pH 2.5; eluent B: 20% 50 mM phosphoric acid, 80%
10   methanol, pH 2.5; gradient: 0-8 min 100% A, 8-13 min 0% A,
13-19 min 100% A). The elution rate was set at
1.0 ml·min^{-1}; the column temperature was set at 40°C.
Post-column derivatization was carried out using
o-phthalic dialdehyde in a reaction capillary (14 m ·
15   0.35 mm) at room temperature. L-phenylalanine was found to
have a retention time of 6.7 min under the conditions
described.

Measurement of the glucose concentration with
enzyme test strips (Diabur, Boehringer Mannheim, Germany)
20   and, depending on the results, the subsequent addition of
2 ml of a concentrated glucose solution (500 g·l^{-1})
ensured that glucose limitation did not arise in the
experimental mixtures. After an incubation time of 48 h, a
(phenylalanine) index value of 119 was achieved after
25   inducing the host strain E. coli AT2471; this compares
with a phenylalanine value of 100 for the uninduced host
strain. Simply introducing the plasmid pZY557tkttal into
the host strain had the result of increasing this index
value, describing the phenylalanine concentration, to 167
30   even without inducing the cells. As the experiments show,
inducing the strain E. coli AT2471/pZY557tkttal resulted
in a further increase to a value of 204. This corresponded
to an increase of 71% as compared with the induced host
strain.

This result demonstrates the positive effect
according to the invention, which effect increases the
synthesis of aromatic compounds, of additionally
increasing the activity of a transketolase in strains
5   which already have an increased transaldolase activity, or
of additionally increasing the activity of the
transaldolase in strains which already have an increased
transketolase activity.

10   Example 5
Production of substances using strains having an increased
transaldolase activity
        The mutant *E. coli* AT2471/pZY557tal was cultured
in an experiment which was otherwise identical to that
15   described in Example 4. After 48 h, induction of the
strain resulted in a (phenylalanine) index value of 131,
which compares with a phenylalanine value of 100 for the
uninduced strain.
        This result clearly demonstrates the positive
20   effect which increasing the activity of a transaldolase
exerts on the synthesis of aromatic substances,
particularly after inducing in accordance with the
invention.

25   Example 6
Production of substances using PTS⁻ mutants in which a
PEP-independent sugar uptake system is being expressed in
addition to there being an increased transaldolase
activity, with the transaldolase activity being increased
30   after the introduction of the PEP-independent sugar-uptake
system.
        The *glf* gene was obtained, as described by
Weisser P. et al. (J. Bacteriol. 177 (1995) 3351-54) using
PCR (Mullis K.B. et al., Meth. Enzymol. 155 (1987) 335-

50). The complete nucleotide sequence of this gene is
available (Barnell W.O. et al., J. Bacteriol. 172 (1990)
7227-40). The *glf* gene was amplified using plasmid pZY600
as the template (Weisser P. et al., J. Bacteriol 177

5   (1995) 3351-4).

        For the purpose of integrating the *glf* gene into
genes which encode components of the *E. coli* PTS system,
plasmid pPTS1 (see Table 1) was digested with *Bgl*II and
treated with Klenow fragment. The unique cleavage site is

10 located in the *pts*I gene. The *glf* gene was isolated, as a
*Bam*HI-*Kpn*I fragment, from plasmid pBM20glfglk and likewise
treated with Klenow fragment. Clones carrying the *glf* in
the same orientation as the *pts*HI genes were obtained by
blunt-end ligation. A 4.6 kb *Pst*I fragment carrying the 3'

15 region of the *pts*H gene and *pts*I containing integrated *glf*
and *crr* was obtained from the resulting plasmid pPTSglf.
This fragment was ligated into the *Eco*RV cleavage site of
vector pGP704. Since this vector is only able to replicate
in λ*pir* strains, the vector has been integrated into the

20 chromosome by transformants which do not harbour this
phage if these transformants are able to grow on
carbenicillin. The integration was checked by Southern
blot analysis (Miller V.L. et al., J. Bacteriol. 170
(1988) 2575-83). The resulting transformants contained the

25 complete PTS genes in addition to the *glf* gene.

        The vector moiety can be recombined out in a
second homologous crossover, resulting in the loss of the
carbenicillin resistance. Since, in this case, the *pts*
genes are interrupted by the insertion of the *glf* gene,

30 the PTS is not expressed in a functional manner in these
mutants. The desired PTS⁻ mutants were selected as
follows: after subculturing the transformants, which were
still PTS⁺, several times on LB medium without
antibiotics, aliquots of the cell suspension were plated

out on LB plates containing 100 µg·l^{-1} phosphomycin. PTS$^-$ mutants are able to grow on these plates. Growing clones were streaked out on LB plates containing either phosphomycin or 20 µg·l^{-1} carbenicillin. Chromosomal DNA was

5 isolated from clones which still exhibited growth on the phosphomycin plates but which were not able to grow on the carbenicillin plates. The integration of the *glf* gene into the genes which encode the PTS system was confirmed by Southern analysis. Corresponding mutants were identified

10 as being phenotypically PTS-deficient. One clone was selected as the host organism *E. coli* AT2471glfintPTS$^-$ and used for the transformations (see above) with plasmid pZY557tal. Following the experimental conditions described for Examples 4 and 5, the PTS-negative mutant *E. coli*

15 AT2471glfintPTS$^-$/pZY557tal, and the corresponding host strain AT2471glfintPTS$^-$, were in each case cultured in two parallel mixtures and the cells of one mixture in each case were induced after approx. 7 divisions.

The induction reduced the synthetic performance

20 of the host strain, which initially had an index value of 100 without induction, down to a value of 56. By comparison, the induction increased the phenylalanine index value from 73 to 103, and consequently above the initial value for the host strain, in cultures of the

25 transformed strain AT2471glfintPTS$^-$/pZY557tal.

This result demonstrates that simply increasing the transaldolase activity has a positive effect on the synthesis of phenylalanine even in those microorganisms in which the activity of the PTS system is diminished, or

30 this system is completely switched off, and into which, at the same time, a PEP-independent sugar uptake system has been integrated.

Example 7

Production of substances using PTS⁻ mutants in which in addition to an increased transaldolase activity a PEP-independent sugar-uptake system has been expressed, with the transaldolase activity being increased before the
5   introduction of the PEP-independent sugar-uptake system.

    E. coli AT2471/pZY557tal was obtained as described in Example 1. Subsequently in this strain a *glf* gene was integrated into the genes which encode components of the PTS system of *E. coli*. This integration was carried
10 out as described in Example 6. Using the experimental conditions described in Examples 4 and 5, these PTS-negative *E.coli* AT2471glfintPTS⁻/pZY557tal mutants were cultured. It was found that the biosynthetic performance of the derived mutants corresponded to that of the strains
15 described in Example 6.

    Example 8
    Production of substances in a fermentation vessel using strains having an increased transaldolase activity and
20 strains having an increased transketolase activity in addition to their increased transaldolase activity.

    The mutants *E. coli* AT2471, *E. coli* AT2471/pZY557tal and *E. coli* AT2471/pZY557tkttal were cultured in a fermentation vessel (reactor volume 15 l).
25 To this end, two shaking flasks (2000 ml) with the mineral medium described in Example 2 (250 ml) were used for preculturing. In this process, the glucose concentration was reduced to 5.0 g.l⁻¹. The flasks were inoculated with 2 ml of glycerol culture and incubated on an orbital
30 shaker at 37°C and 150 rpm until an optical density (600 nm) of 3 was reached.

    The precultures were used for inoculating 4.5 l of production medium. This contained $MgSO_4 \cdot 7H_2O$ (0.3 g·l⁻¹), KH_2PO_4 (3.0 g·l⁻¹), NaCl 0.1 (g·l⁻¹), $(NH_4)_2SO_4$ (5.0 g·l⁻

^{1}), $CaCl_2 \cdot 2H_2O$ (15.0 mg·l^{-1}), $FeSO_4 \cdot 7H_2O$ (0.75 gl^{-1}) and L-tyrosine (0.24 g·l^{-1}). Additional minerals were added in the form of a trace element solution (1.5 ml·l^{-1}), which was composed of $Al_2(SO_4)_3 \cdot 18H_2O$ (2.0 g·l^{-1}), $CoSO_4 \cdot 6H_2O$

5   (0.7 g·l^{-1}), $CuSO_4 \cdot 5H_2O$ (2.5 g·l^{-1}), H_3BO_3 (0.5 g·l^{-1}), $MnCl_2 \cdot 4H_2O$ (20.0 g·l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (3.0 g·l^{-1}), $NiSO_4 \cdot 3H_2O$ (2.0 g·l^{-1}) and $ZnSO_4 \cdot 7H_2O$ (15.0 g·l^{-1}). Vitamin B1 (75.0 mg·l^{-1}) was dissolved in water and added, after having been sterilized by filtration, to the medium after

10 the latter had been autoclaved. Glucose (15 g·l^{-1}) was autoclaved separately and likewise added to the medium after the latter had been autoclaved. By adding NH_4OH (25%) the pH value in the medium was controlled at 7, the oxygen partial pressure being controlled at a value of 20%

15 via the stirrer speed, the air volume fed and the pressure inside the fermentation vessel. By adding a glucose solution (700 g·l^{-1}) the glucose concentration in the culture medium was kept at values around 5 g.l^{-1}. Depending on the fermentation time, L-tyrosine was added

20 to the glucose solution in variable concentrations in order to avoid L-tyrosine limitation.

        In experiments in which cell induction was effected, this was done in accordance with the invention after 6 hours by adding 50 μM of IPTG. After an incubation

25 time of 36 hours, a (phenylalanine) index value of 120 was achieved by simply introducing the pZY557tal plasmid; this compares with a (phenylalanine) index value of 100 for the uninduced host strain *E. coli* AT2471. By means of induction according to the invention this value could be

30 raised further to 153. By introducing the pZY557tkttal plasmid alone and thus raising the transketolase activity in addition to the transaldolase activity, a (phenylalanine) index value of 130 could be achieved compared with the host strain. The experiments further

showed that the induction of the strain *E. coli*
AT2471/pZY557kttal enabled a further increase in the
(phenylalanine) index value to 250. This corresponds to an
increase of 92% compared with the uninduced strain.

5   This result shows that even in experiments
carried out in fermentation vessels an increase in the
synthesis of aromatic compounds can be achieved according
to the invention by increasing the transaldolase activity
as well as by increasing the transketolase activity in
10 strains which already have an increased transaldolase
activity.

Table 1:

Strains and plasmids	Genotype/ characteristics	Source or reference
E.coli AT2471	*tyr*A4, *rel*A1, *spo*T1, *thi*-1	Taylor and Trotter, Bacteriol. Rev. 13 (1967) 332-53
E. coli SY327	araD, Δ(lac-*pro*), Rif^r, recA56, λ-phage *pir* function	Miller et al., J. Bacteriol. 170 (1988) 2575-83
E. coli CC118	Δ(*ara-leu*), araD, ΔlacX74, *galE*, *galK*, phoA20, *thi*-1, *rpsE*, *rpoB*, *argE* (Am), *recA1*, λ*pir* lysogen	Manoil et al., Proc. Natl. Acad. Sci USA 82 (1985) 8129-33
pZY507	Cm^r	Weisser et al., J. Bacteriol 177 (1995) 3351-4
pZY507glfglk	Z. mobilis *glf* and glk genes in p-ZY507	Weisser et al., J. Bacteriol 177 (1995) 3351-4
pZY557	pZY507, multiple cloning site of pUCBM20, Cm^r	Sprenger, unpublished
pZY557tkttal	*E. coli* *tkt*A and *tal*B genes in pZY557	This patent application

Strains and plasmids	Genotype/ characteristics	Source or reference
pACYC184	Cm^r, Tet^r	Chang and Cohen, J. Bacteriol. 134 (1978) 1141-1156
pDIA3206	Ap^r, 11.5 kb insert from *E. coli* K12 chromosome including *ptsHI-crr* genes	DeReuse et al., J. Bacteriol. 170 (1988) 3827-37
pPTS1	pACYC184 containing a 4 kb *ClaI* fragment which contains the *ptsHI* and *crr* genes from pDIA3206; Cm^r	Jahreis, Osnabruck university, unpublished
pGP704	Amp^r	Miller et al., J. Bacteriol. 170 (1988) 2575-83

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Table 2

Microorganism	Time after induction/ h	Transaldolase activity/ U·(mg of protein)$^{-1}$	
		without induction	with induction
E. coli AT2471/	0		0.64
E. coli AT2471/ pZY557	0 3		0.65 0.80
E. coli AT2471/ pZY557tal	0 3	0.53 0.6	0.61 1.06
E. coli AT2471/ pZY557tkttal	0 3	0.70 0.45	0.80 1.16

C L A I M S

1. Process for the microbial preparation of substances
   in which the activity of a transaldolase is increased
   in a microorganism which is producing these
   substances, with the activity of a
   phosphoenolpyruvate (PEP)-dependent sugar-uptake
   system in this microorganism being present, reduced
   or absent.

2. Process according to Claim 1, characterized in that
   the preparation of substances is effected in
   microorganisms which have not, prior to the
   increasing of the transaldolase activity, been
   selected as strains with a switched-off
   phosphotransferase system (pts⁻ strains) from strains
   which previously harboured a phosphotransferase
   system (pts⁺ strains).

3. Process according to Claim 1 or 2, characterized in
   that substances are prepared in whose synthesis at
   least one intermediate of the pentose phosphate
   pathway is involved.

4. Process according to Claim 3, characterized in that
   the intermediate is erythrose 4-phosphate (Ery4P).

5. Process according to Claim 1 or 4, characterized in
   that the activity of an *Escherichia coli*
   transaldolase is increased.

6. Process according to one of Claims 1 to 5,
   characterized in that the activity of *Escherichia
   coli* transaldolase B (TalB) is increased.

7. Process according to one of Claims 1 to 6,
   characterized in that the activity of a transketolase
   is additionally increased.

8. Process according to Claim 7, characterized in that
   the activity of an *Escherichia coli* transketolase is

increased.

9. Process according to one of Claims 7 or 8, characterized in that the activity of *Escherichia coli* transketolase A (TktA) is increased.

10. Process according to one of Claims 1 to 9, characterized in that the activity of a transport protein for the PEP-independent uptake of a sugar and/or the activity of a kinase which phosphorylates the relevant sugar are/is additionally increased.

11. Process according to Claim 10, characterized in that the transport protein is a facilitator.

12. Process according to Claim 11, characterized in that the facilitator is the *Zymomonas mobilis* glucose facilitator protein (Glf).

13. Process according to one of Claims 10 to 12, characterized in that the kinase is a hexose-phosphorylating kinase.

14. Process according to Claim 13, characterized in that the kinase is derived from *Zymomonas mobilis*.

15. Process according to Claim 14, characterized in that the kinase is *Zymomonas mobilis* glucokinase (Glk).

16. Process according to one of Claims 10 to 15, characterized in that the activity of a PEP-dependent sugar uptake system, if present, is additionally decreased or switched off.

17. Process according to one of Claims 1 to 16, characterized in that the activity of at least one of the following components, i.e. transaldolase, transketolase, transport protein and kinase, is increased

    a)   by introduction of the genes

    b)   and/or by increasing the gene copy number

    c)   and/or by increasing gene expression

    d)   and/or by increasing the endogenous activity of

the said enzymes
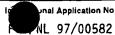
   e)   and/or by altering the structure of the enzymes

   f)   and/or by using deregulated enzymes

   g)   and/or by inserting genes which encode

       deregulated enzymes.

18.   Process according to Claim 17, characterized in that the increase in the activity is achieved by integrating the gene or the genes into a gene structure or into several gene structures, with the gene or the genes being introduced into the gene structure as (a) single copy(ies) or in increased copy number.

19.   Process according to one of Claims 1 to 18, characterized in that a microorganism is employed in which one or more enzymes, which are additionally involved in the synthesis of the substances, are deregulated and/or exhibit increased activity.

20.   Process according to Claims 1 to 19, characterized in that the substance which is prepared is an aromatic amino acid.

21.   Process according to Claim 20, characterized in that the aromatic amino acid is L-phenylalanine.

22.   Process according to one of Claims 1 to 21, characterized in that the microorganism employed belongs to the genus *Escherichia, Serratia, Bacillus, Corynebacterium* or *Brevibacterium*.

23.   Process according to Claim 22, characterized in that the microorganism is *Escherichia coli*.

24.   Gene structure containing, in recombinant form,

   a)   a gene encoding a transaldolase or a gene encoding a transaldolase and a gene encoding a transketolase, and

   b)   at least one gene encoding a transport protein for the PEP-independent uptake of a sugar or encoding a sugar-phosphorylating kinase.

25. Gene structure according to Claim 24, characterized in that the gene for the transport protein encodes a facilitator and the gene for the kinase encodes a hexose-phosphorylating kinase.

26. Gene structure according to Claim 24 or 25, characterized in that the genes for the transaldolase and the transketolase are derived from *Escherichia coli* and the genes for the transport protein and the kinase are derived from *Zymomonas mobilis*.

27. Gene structure according to Claims 24 to 26, characterized in that the gene for the *Escherichia coli* transaldolase is *talB* and the gene for the *Escherichia coli* transketolase is *tktA*, and the gene for the *Zymomonas mobilis* transport protein is *glf* and the gene for the *Zymomonas mobilis* kinase is *glk*.

28. Gene structure according to one of Claims 24 to 27, characterized in that the gene structure contains at least one regulatory gene sequence which is assigned to one of the genes.

29. Gene structure according to Claim 28, characterized in that at least one of the genes in the gene structure is incorporated such that it is under the control of an inducible promoter.

30. Transformed cell, harbouring, in replicable form, a gene structure according to Claims 24 to 29.

31. Transformed cell according to Claim 30, characterized in that, in the cell, one or more enzymes which are additionally involved in the synthesis of the substances are deregulated and/or exhibit increased activity.

32. Transformed cell according to Claim 30 or 31, characterized in that the cell is an *Escherichia coli* cell.

33. Transformed cell according to one of Claims 30 to 32,

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characterized in that the activity of the PEP-dependent sugar uptake system, if this system is present, is decreased or switched off.

34. Transformed cell according to one of Claims 30 to 33, characterized in that it is able to produce an aromatic amino acid.

35. Transformed cell according to Claim 34, characterized in that the aromatic amino acid is L-phenylalanine.

36. Process for the microbial preparation of substances according to one of Claims 1 to 23, characterized in that transformed cells according to one of Claims 30 to 35, in which there is a gene structure according to Claim 29, are cultured, with the induction being effected after 2 cell divisions at the earliest.

37. Process according to Claim 36, characterized in that, in addition to the intermediates of the pentose phosphate pathway, the transformed cell also contains other metabolites of central metabolism in increased availability.

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/54   C12P13/22   C12P19/02   C12P1/00   C12N15/31
        C07H11/04   C12N1/21   //C12Q1/48,(C12N1/21,C12R1:07,1:13,
        1:15,1:185,1:19,1:425)

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C12P   C07K   C07H   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIAO J.C. ET AL.: "Pathway analysis, engineering, and physiological considerations for redirecting central metabolism" BIOTECHNOLOGY AND BIOENGINEERING, vol. 52, no. 1, 5 October 1996, pages 129-140, XP002053640 cited in the application	1-4,7-9, 17-23
Y	see abstract	5,6, 10-16, 24-37
	see page 130, right-hand column, paragraph 4 see page 132 - page 133	

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1998	20/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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International Application No

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Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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A	BERRY A.: "Improving production of aromatic compounds in Escherichia coli by metabolic engineering" TRENDS IN BIOTECHNOLOGY, vol. 14, no. 7, July 1996, page 250-256 XP004035764 see page 252, right-hand column, paragraph 2 - page 255, right-hand column, paragraph 1	1-10,13, 15-37
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